Exhibit II

Self-Reporting Arabidopsis Expressing pH and [Ca^{2+}] Indicators Unveil Ion Dynamics in the Cytoplasm and in the Apoplast under Abiotic Stress[1][w]

Dongjie Gao, Marc R. Knight, Anthony J. Trewavas, Burkhard Sattelmacher, and Christoph Plieth*

Institut für Pflanzenernährung und Bodenkunde, Christian-Albrechts-Universität, D–24098 Kiel, Germany (D.G., B.S.); Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, United Kingdom (M.R.K.); Institute of Cell and Molecular Biology (Botany), Mayfield Road, Edinburgh EH9 3JH, United Kingdom (A.J.T.); and Zentrum für Biochemie und Molekularbiologie, Universität Kiel, Am Botanischen Garten 9, 24118 Kiel, Germany (C.P.)

For noninvasive in vivo measurements of intra- and extracellular ion concentrations, we produced transgenic Arabidopsis expressing pH and calcium indicators in the cytoplasm and in the apoplast. Ratiometric pH-sensitive derivatives of the green fluorescent protein (At-pHluorins) were used as pH indicators. For measurements of calcium ([Ca^{2+}]), luminescent aequorin variants were expressed in fusion with pHluorins. An Arabidopsis chitinase signal sequence was used to deliver the indicator complex to the apoplast. Responses of pH and [Ca^{2+}] in the apoplast and in the cytoplasm were studied under salt and "drought" (mannitol) stress. Results are discussed in the frame of ion flux, regulation, and signaling. They suggest that osmotic stress and salt stress are differently sensed, compiled, and processed in plant cells.

How plants sense and respond to various environmental stimuli, such as touch, wind, cold, light, oxidative stress, high salinity, and drought, has become an area of intense investigations in the past decades. Many cellular compounds such as Ca^{2+}, lipids, H^+, cyclic nucleotides, and inositolphosphates are listed as messengers used by plants to forward and compile cellular signals (Sanders et al., 1999).

The cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$), in particular, has been found to play a central role in a wide range of cellular events (Trewavas and Malhó, 1998; Knight, 2000; Sanders et al., 2002), although its alleged universality has been critically questioned recently (Plieth, 2001; Scrase-Field and Knight, 2003).

The proton activity ([H^+]) is an extremely important factor as well. [H^+] is involved in cell signaling either directly or in cross talk with plant hormones or calcium (Gilroy and Trewavas, 1994; Ward et al., 1995; Blatt and Grabov, 1997; Roos, 2000; Felle, 2001). Therefore, the cells exert tight control over [H^+] (Felle, 1987).

The apoplast is the first plant compartment encountering environmental signals. It has been suggested that the apoplast is involved not only in the response but also in the perception and transduction of various environmental signals in cooperation with the plasma membrane (Hoson, 1998). pH and calcium concentrations in the apoplast have been measured in many plants by selective electrodes (Zieschang et al., 1993; Felle, 1998), by collecting apoplast fluid (Dannel et al., 1995; Mühling and Sattelmacher, 1995; Cramer and Jones, 1996; Lohaus et al., 2001) or by impermeable fluorescent dyes (Hoffmann and Kosegarten, 1995; Mühling et al., 1995; Taylor et al., 1996; Sakurai, 1998a, 1998b). However, little in vivo information on the change of apoplastic pH and [Ca^{2+}] is available from whole and undisturbed (i.e. not infiltrated) plants under abiotic stress. Such knowledge can help to understand the role of the apoplast in more detail. To overcome drawbacks of other methods, we started to develop a novel approach: the design of transgenic plants that simultaneously express their own [Ca^{2+}] and pH indicators at the desired location. We expressed modified pHluorins (Miesenböck et al., 1998; Moseyko and Feldman, 2001; Plieth et al., 2001) and aequorin (AQ; Knight et al., 1991, 1997a) as fusion proteins in the cytoplasm of Arabidopsis (Fig. 1, A, E, and F). For studying extracellular ion dynamics, we also targeted pHluorins and an AQ variant with low calcium affinity (Kendall et al., 1992) as fusion proteins to the apoplast by using an Arabidopsis chitinase signal sequence (Fig. 1, B–D). A series of experiments were performed to characterize the indicators (Figs. 2–4) and to demonstrate that the produced self-reporting plants are able to monitor changes of pH and [Ca^{2+}]

[1] This work was supported by the Deutsche Forschungsgemeinschaft (grant nos. SA 359/12–3 to B.S. and D.G. and PL253/1–1 to C.P.) and by the European Commission (grant no. BIO4–CT97–5080 to C.P.).

[w] The online version of this article contains Web-only data.

* Corresponding author; e-mail cplieth@zbm.uni-kiel.de; fax 49–431–880–4368.

http://www.plantphysiol.org/cgi/doi/10.1104/pp.103.032508.

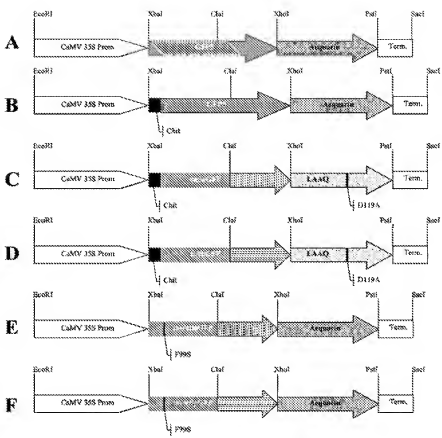

Figure 1. Schematic structures of the gene cassettes. A, Fusion construct from pCM2. B, Apoplast targeting construct: insertion of chitinase signal sequences (66 bp) upstream of GFP5 giving p*chit*GFP5:AQ. C, Fragment exchange (Cla-Xho) to yield pH sensitivity and (*Xho*I-*Pst*I) for insertion of low-affinity AQ (LAAQ), leading to p*chit*ratioGFP:LAAQ. D, Same as C but insertion of ecliptic pHluorin fragment giving p*chit*eclipGFP:LAAQ. E and F, Replacement of Cla-Xho fragment for introducing pH sensitivity and introduction of point mutation for increased solubility. This produced cytoplasmic expression of ratioGFP (E) and ecliptic green fluorescent protein (GFP; F), i.e. p*sm*ratioGFP:AQ and p*sm*eclipGFP:AQ, respectively.

in the cytoplasm and in the apoplast (Figs. 5–7). Salt (NaCl) and drought stress were studied in particular with this novel technique (Figs. 6–10) to discover new aspects of ion flux, regulation, and signaling.

RESULTS

Before expression in plants, recombinant indicators were expressed in bacteria and properties verified in vitro.

Characterization of AQ Variants in Bacteria

For comparison of native AQ and LAAQ, both AQ variants were expressed in bacteria. Bacteria were reconstituted at room temperature for 30 min with 10 μM coelenterazine (CTZ) in Luria-Bertani medium and then stimulated with 1 mM $CaCl_2$ solution. The kinetics (data not shown) were similar to what has been shown previously (Jones et al., 2002). Relative luminescence of initial response was lower in LAAQ-expressing bacteria in comparison with native AQ. The measured relative luminescence indicates that less LAAQ than AQ is discharged during Ca^{2+} entry into the cells. This verifies that LAAQ is, because of its lower Ca^{2+}-binding affinity, the better choice for a higher $[Ca^{2+}]$ range as is expected in the apoplast.

Characterization and in Vitro Calibration of Modified pHluorins

Modified At-pHluorins isolated from bacteria were characterized fluorometrically. Ratiometric At-pHluorin (ratioGFP) has pH-dependent spectra with isoexcitation point at 428 nm (Fig. 2A). The excitation maxima of ratioGFP are at 395 and 475 nm, and the emission maximum is at 508 nm. Its k_d is 6.73 ± 0.03, and its optimal dynamic range is in the interval 5.6 < pH <7.8, which makes it suitable for both cytoplasmic and apoplastic pH measurements (Fig. 2B).

Ecliptic At-pHluorin (eclipticGFP) displayed a ratiometric behavior in the emission mode with an isoemission point at 490 nm (Fig. 3A). The excitation maxima of eclipticGFP are at 398 and 477 nm, and the emission maximum is at 510 nm. Its k_d is 7.25 ± 0.02, and its optimal dynamic range is in the interval 6.5 < pH < 8.0, which makes it suitable especially for cytoplasmic pH measurements (Fig. 3B).

The spectra (Figs. 2 and 3) show that all modifications made to produce At-pHluorins hardly altered spectral properties and/or pH dependency when compared with original pHluorins from Miesenböck et al. (1998). In addition, when fused to AQ, the emission spectra were negligibly broadened by 3 nm in half band width, and peaks were shifted by about 1 nm toward the blue. Also, the pH dependence of

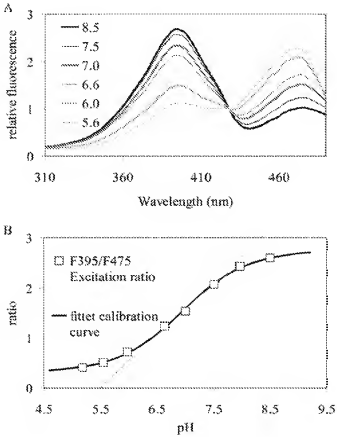

Figure 2. A, Excitation spectra of ratiometric GFP at different pHs (emission wavelength = 508 nm). Curves were normalized by F(428ex; 508em). B, Corresponding calibration curve for fluorescence excitation ratio R(395ex/475ex; 508em); dissociation constant, i.e. midpoint of the fitted curve = $pK_d = 6.9 \pm 0.03$; optimal dynamic range for pH measurements is between 5.6 and 8.0.

At-pHluorins did not significantly alter when fused with AQ and chitinase propeptide. Moreover, the NaCl concentration (0–200 mM NaCl) at constant pH had negligible influence on the fluorescence ratios (data not shown).

Luminescence. In Vivo Reconstitution of AQs

Plants expressing the GFP5:AQ fusion in the cytoplasm and in the apoplast were in vivo reconstituted with two different CTZ derivatives. Figure 4 shows absolute luminescence during the first hours of reconstitution. The time courses demonstrate that maximal basal luminescence in the cytoplasm (Fig. 4A) is reached after 4 h. The apparent binding constant of cp-AQ is $pk_{Ca} \sim 6.4$ compared with $pk_{Ca} \sim 5.9$ of native AQ (Plieth and Trewavas, 2002). As a consequence, basal luminescence produced by cp-AQ decays, even with the low resting level of free $[Ca^{2+}]$ in the cytoplasm (Fig. 4A, circles), whereas basal luminescence from native AQ is stable here (Fig. 4A, squares). A much higher $[Ca^{2+}]$ is present in the apoplast. Hence, even luminescence from native AQ decays and cp-AQ luminescence dissipates within 5 h in this compartment (Fig. 4B). We found that basal luminescence even from LAAQ is not stable in the apoplast when reconstituted with native CTZ and also decays below minimum detection level within 2 d (data not shown). Nevertheless, there is a time window of more than 12 h for $[Ca^{2+}]$ measurements after in vivo reconstitution of targeted AQ with native CTZ.

$[Ca^{2+}]$ Response to Cold in the Cytoplasm and in the Apoplast

The in vivo response of luminescence from AQ expressed in the apoplast to cooling was compared with luminescence responses reported by AQ expressed in the cytoplasm (Fig. 5). These responses, too, show that AQ has successfully been targeted to a compartment different from the cytoplasm. At first glance, the data suggest that $[Ca^{2+}]$ is slightly lowered in this compartment during cold period. However, AQ luminescence is temperature dependent (data not shown), and the decrease of luminescence

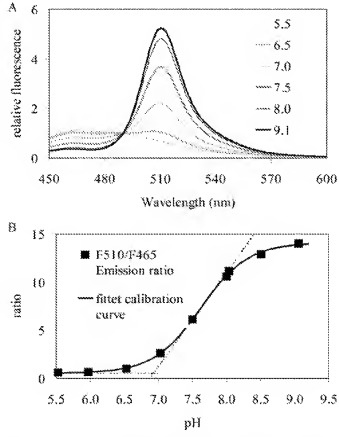

Figure 3. A, Fmission spectra of ecliptic GFP at different pHs (excitation wavelength = 400 nm). Curves were normalized by F(400ex; 490em). B, Corresponding calibration curve for fluorescence emission ratio R(400ex; 490em/510em); dissociation constant, i.e. midpoint of the fitted curve = $pK_d = 7.25 \pm 0.02$; optimal dynamic range for pH measurements is between 6.5 and 8.0.

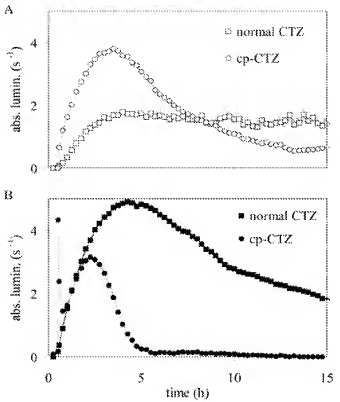

Figure 4. Development of basal AQ luminescence during in vivo reconstitution of Arabidopsis roots with different coelenterazine (CTZ) derivatives. Squares, Reconstitution with native CTZ; circles, reconstitution with cp-CTZ. Addition of CTZ at t = 0.6 h to 10 μM final concentration. Note: Absolute luminescence (×10,000) is given here, which is luminescence of each integration interval divided by total luminescence produced by the specimen. Relative luminescence (×10,000) is given in all other figures, which is luminescence of each integration interval divided by luminescence still remaining in the specimen. A, Reconstitution of AQ expressed in the cytoplasm. B, Reconstitution of AQ targeted to the apoplast.

with lowered temperature in Figure 5C might well originate from this effect.

Fluorescence. Expression and Localization of the Indicator Complex

The expression level of indicator protein—as scrutinized by fluorescence—seemed to be higher in the apoplast expressing lines than in the cytoplasmic-expressing lines. This can be explained with the permanent export of indicator in apoplast-targeted lines and extracellular accumulation. Confocal laser scanning microscopy (CLSM) revealed staining of the cell wall (supplemental data).

The in vivo spectra taken from ratiometric pHluorins expressed in roots (Fig. 6) are slightly different compared with the in vitro spectra (Fig. 2) because of slight autofluorescence in the plant specimen and different emission bands (fluorescence emission wavelength F_{em} = 508 ± 5 nm in Fig. 2 and F_{em} = 535 ± 25 nm in Fig. 6). However, the spectra clearly indicate compartments of different acidity: pH ≈ 6.3 in the apoplast and pH ≈ 7.2 in the cytoplasm.

A change of external pH (Fig. 7) induced no change in fluorescence ratio of cytoplasmic-expressed At-pHluorin. The pH indicator expressed in the apoplast, in contrast, reported a strong dependence of apoplastic pH on external pH (Fig. 7). This demonstrates that the indicator complex is located in a compartment that has direct access to the outer medium and confirms successful targeting of the protein to the extracellular space.

Free Calcium Concentration in the Apoplast ($[Ca^{2+}]_{apo}$) and Cytoplasmic Free Calcium Concentration ($[Ca^{2+}]_{cyt}$) Responses during "Drought" and NaCl Stress

Changes of $[Ca^{2+}]_{apo}$ and $[Ca^{2+}]_{cyt}$ were determined in response to repeated periods of NaCl stress

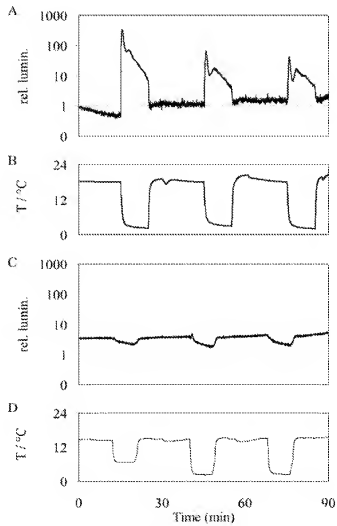

Figure 5. Luminescence response to cold from Arabidopsis expressing AQ in the cytoplasm (A and B) and in the apoplast (C and D). A and C, Relative luminescence; B and D, corresponding temperature measured in parallel.

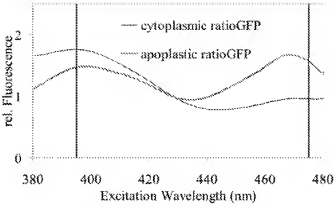

Figure 6. In vivo spectra of ratioGFP expressed in Arabidopsis roots (black curve, cytoplasmic expression; gray curve, apoplastic expression). Curves were normalized by F(428ex; 530em).

(i.e. 100 mM NaCl) and "drought." The latter was mimicked by isosmotic mannitol.

Osmotic ("Drought") Stress

Repeated 30-min periods of mannitol treatment were applied to roots. The results (Fig. 8A) indicate that the $[Ca^{2+}]_{apo}$ is hardly affected by this treatment (Fig. 8A, gray curve). Apart from tiny responses during mannitol washout, there are no significant changes in the $[Ca^{2+}]_{apo}$ signal. The $[Ca^{2+}]_{cyt}$, in contrast, is drastically affected, and pronounced $[Ca^{2+}]_{cyt}$ transients are observed (Fig. 8A, black curve). This experiment produces two different kinds of stimuli: first, a hyperosmotic stimulus when mannitol is applied and second a hypo-osmotic stimulus when mannitol is washed out. The very first hyperosmotic treatment is a weak stimulus giving a $[Ca^{2+}]_{cyt}$ transient with small amplitude (at t = 0.5 h; Fig. 8, A and C). All following hyperosmotic treatments (t = 1.5, 2.5, and 3.5 h; Fig. 8A) do not give spectacular $[Ca^{2+}]_{cyt}$ transients anymore, suggesting that some kind of adaptation must have happened. The hypo-osmotic-triggered $[Ca^{2+}]_{cyt}$ response, however, is much more pronounced indicating that this stimulus is more critical. In both cases (hyper- and hypo-osmotic shock), the $[Ca^{2+}]_{cyt}$ is brought back to base level by cellular Ca^{2+} clearance mechanisms after the transient. $[Ca^{2+}]_{cyt}$ does not stay on elevated levels as is the case for NaCl treatment (described below).

NaCl Stress

Repeated periods of NaCl treatment give drastic transients and prolonged alterations of the $[Ca^{2+}]$ level in both compartments (Fig. 8, B and D). The very first NaCl treatment leads to a permanent increase of the cytoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_{cyt}$) after a short transient peak (Fig. 8D). The following washout, however, produces a further permanent increase (Fig. 8B) that is not seen during mannitol treatment (Fig. 8A). These two increased $[Ca^{2+}]_{cyt}$ levels alternate during the rest of the experiment as NaCl solution is replaced by water and vice versa. The $[Ca^{2+}]_{apo}$ is markedly affected by NaCl as well (Fig. 8B, gray curve). During the very first NaCl treatment, a distinct increase in $[Ca^{2+}]_{apo}$ occurs. The following treatments bring also about an increase or at least an elevated steady state of $[Ca^{2+}]_{apo}$ during NaCl periods and a decrease during washout (water). To discriminate NaCl stress and osmotic response, we switched between mannitol and NaCl solutions with same osmotic pressure (Fig. 9). This brings about a uniform osmotic stress. The very first transition from mannitol to NaCl (t = 1.5 h; Fig. 9) resulted in an elevation of $[Ca^{2+}]_{cyt}$ as was observed before (t = 0.5 h; Fig. 8B), but with a negligible transient peak. The subsequent change from NaCl to mannitol (t = 2 h; Fig. 9) unexpectedly produced a $[Ca^{2+}]_{cyt}$ transient, but no change of the reached steady-state level. All subsequent transitions from mannitol to NaCl and vice versa caused similar alterations between two $[Ca^{2+}]_{cyt}$ states—with intermediate transients—as has been observed with NaCl-water treatment (Fig. 8). The same treatment of the apoplastic expressing plants (Fig. 9, gray curve) confirmed that external Na^+ caused a drastic increase in $[Ca^{2+}]_{apo}$ and gave a similar kinetic pattern as with NaCl-water treatment (Fig. 8B, gray curve).

Cytoplasmic pH (pH_{cyt}) and Apoplastic pH (pH_{apo}) Responses during NaCl and "Drought" Stress

The F_{395} to F_{475} fluorescence excitation ratio of the pH-sensitive GFP revealed that "drought" (mannitol) treatment (Fig. 10A) does not influence pH_{cyt} and pH_{apo}. NaCl stress, however, resulted in significant alterations of pH in the apoplast and to a minor extent in the cytoplasm (Fig. 10B). The pH responses

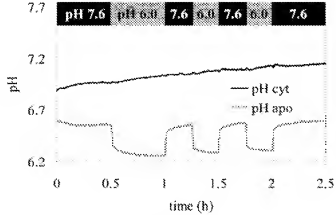

Figure 7. pH changes in the apoplast (gray curve) and the cytoplasm (black curve) of Arabidopsis roots in response to changes in external pH. External pH was adjusted with 10 mM HEPES (pH 7.6) and 10 mM MES (pH 6.0) in standard medium (KCl, $CaCl_2$, and $MgCl_2$: 1 mM each).

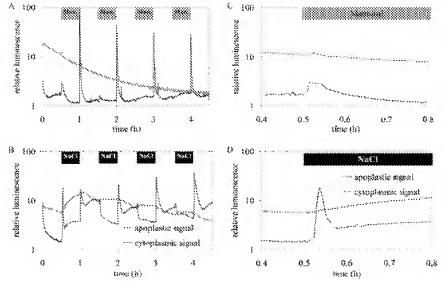

Figure 8. A, Cytoplasmic and apoplastic [Ca^{2+}] in response to "drought" stress (200 mM mannitol versus water) measured by AQ luminescence from Arabidopsis roots. B, Cytoplasmic and apoplastic [Ca^{2+}] in response to NaCl stress (100 mM NaCl versus water). C and D, Close-ups of A, and B, respectively.

became more pronounced with the number of NaCl treatments in both compartments. This sort of "sensitization mechanism" (i.e. increasing response amplitudes with increasing number of treatments) is opposite to "adaptation" mechanisms (i.e. decreasing response amplitudes with increasing number of treatments) usually observed with other abiotic stimuli (e.g. cold; Fig. 5A).

DISCUSSION

For studying ion flux and regulation when the plants are under abiotic environmental stress, genetically encoded indicator proteins proved to be the most elegant way (Plieth, 2001). An important advantage is that they can be targeted to many different organelles, compartments, and tissues by fusion with specific promoters, signal sequences, or by trapped enhancers (Kiegle et al., 2000; Plieth, 2001). When using reporter plants expressing their own indicators, loading problems, as is the case for low-M_r indicator dyes (Plieth and Hansen, 1996), are circumvented, and artifacts resulting from loading procedures are avoided. Measurements can be performed in unperturbed, whole, intact plants and under "physiological" conditions; i.e. conditions similar to what plants experience in the wild. Thus, any study which makes use of "self-reporting plants" relates more closely to conditions in the field and deserves to be called "biologically relevant."

Scrutiny of Transgenic Lines

The fact that none of our transgenic lines differ in phenotype from the wild type verifies that expression of the indicators do not interfere with signal transduction, growth, and development. It suggests that neither cellular ion regulation in general nor ion buffering in particular is affected by the alien Ca^{2+}- and H^+-binding indicator proteins.

Figures 4 to 7 show that the targeted indicator complex is expressed in a compartment with high [Ca^{2+}] and low pH compared with cytoplasmic conditions. Both details have been anticipated for the extracellular space, and there are more details confirming correct targeting of the indicator in the apoplast.

The typical [Ca^{2+}] responses to periods of cold obtained from apoplastic expressing lines (Fig. 5B) are completely different from cytoplasmic [Ca^{2+}] responses (Fig. 5A; Plieth et al., 1999a).

The overall continuing decay of the apoplastic signal (Fig. 8A, gray curve) indicates permanent washout of Ca^{2+} ions by the perifusion medium which was not supplemented with extra Ca^{2+}.

The in vivo spectra (Fig. 6) taken from dissected roots expressing ratioGFP in the cytoplasm (black curve) and in the apoplast (gray curve) are markedly different and their F_{395} to F_{475} ratios indicate $pH_{cyt} \approx 7.2$ and $pH_{apo} \approx 6.3$, which is close to what has been expected. The apoplastic pH has been reported many times from different species, and the majority of values varies between 5.3 (Kosegarten and Englisch, 1994) and 6.7 (Dannel et al., 1995).

However, the measured resting apoplastic pH (i.e. ≈6.3) was always slightly higher than expected (i.e. $pH < 6.0$). Therefore, we reexamined the transgenic lines by CLSM at high magnification (supplemental data). In the cytoplasmic expression lines, cell walls could be easily detected as dark lines between fluorescent protoplasts. The apoplastic expression lines, in contrast, also showed fluorescence at these locations. For a better resolution, the cell walls of these lines were separated from the protoplasts by pretreatment with 100 μM cycloheximide and plasmol-

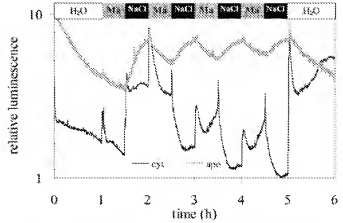

Figure 9. Calcium responses upon drought and NaCl stress (200 mM mannitol versus 100 mM NaCl versus water) in Arabidopsis roots.

ysis with 500 mM mannitol. This finally revealed a clear cell wall staining with GFP.

However, in these lines, we also found residual expression of indicator protein inside the cells located in so-called "fusiform organelles." These organelles are part of the endoplasmic reticulum (ER) network (Hawes et al., 2001). This finding is not surprising because the protein is translocated via the ER because of the preceding N-terminal chitinase signal propeptide (22 amino acids), carried along the secretory pathway, and then excreted to the apoplast because of the missing HDEL retention sequence. So, there is locally always some cellular expression with fluorescence adulterating the global extracellular fluorescence ratio. We found this cellular expression appearing mainly in undifferentiated, meristematic, and growing cells near the root tip and less in the fully differentiated, basal tissue. Therefore, for pH measurements, we chose the fully differentiated tissue of the root hair zone of lateral roots were side effects caused by residual expression in the ER and autofluorescence are negligible. The fact that the "bulk freight" of targeted indicator has reliably been excreted is demonstrated by the test experiment shown in Figure 7: A change of external pH does produce an immediate change of fluorescence ratio. This shows that the main part of indicator, sufficient for reliable pH measurements, is located in the apoplast. Furthermore, there was no At-pHluorin within vacuoles detected by CLSM. This is in contrast to Persson et al. (2002), who also found protein in vacuoles when targeted to ER with C-terminal HDEL sequence removed. For our constructs, this means that either no At-pHluorin has been trafficked to vacuoles or any vacuolar fluorescence has been quenched because of an extremely low pH_{vac}. The latter, however, is unlikely because other studies show that GFP is stably fluorescent even in acidic vacuoles of Arabidopsis (Neuhaus, 2000; Di Sansebastiano et al., 2001; Frigerio et al., 2001).

pH and $[Ca^{2+}]$ Respond Differently to "Drought" and NaCl Stress Situations

The amplitude of the $[Ca^{2+}]_{cyt}$ response to water loss (hyperosmotic stimulus) is much smaller compared with that seen during hypo-osmotic stimulus (Fig. 8A, black curve). This has been observed before (Pauly et al., 2001) and verifies that shrinking is less serious for the cells than swelling, which might lead to rupture if cell wall and/or membrane do not stand the rising pressure (Takahashi et al., 1997). The decrease in $[Ca^{2+}]_{cyt}$ amplitude with repeated mannitol treatment periods (Fig. 8A, black curve) reflects some sort of adaptation that also has been observed with other abiotic stimuli such as cold and gravity (Plieth et al., 1999a; Plieth and Trewavas, 2002). The apoplastic $[Ca^{2+}]$ remains almost unaffected by mannitol treatment (Fig. 8A, gray curve). This is a surprise because we expected that a significant amount of Ca^{2+} ions is shifted from the extracellular space during hypo-osmotic treatment into the cytoplasm. This should give a significant transient fall in the apoplastic luminescence signal. Because the latter fails to

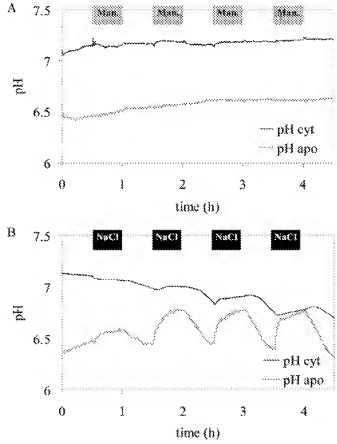

Figure 10. A, Cytoplasmic and apoplastic pH responses upon drought stress (water versus 200 mM mannitol) in Arabidopsis roots measured by fluorescence ratiometry. B, Cytoplasmic and apoplastic pH responses upon NaCl stress (water versus 100 mM NaCl) in Arabidopsis roots.

appear, we conclude that the cellular $[Ca^{2+}]_{cyt}$ transients during mannitol washouts (Fig. 8A, black curve) are mainly produced by release of Ca^{2+} ions from internal stores (e.g. vacuole) rather than influx from the outside.

NaCl, in contrast, produces $[Ca^{2+}]$ and pH changes in both compartments (Figs. 8–10): The finding that $[Ca^{2+}]_{cyt}$ is increased during NaCl stress is in line with observations from Lynch et al. (1989), who observed a $[Ca^{2+}]_{cyt}$ increase in maize (*Zea mays*) root protoplasts during NaCl stress. The finding that $[Ca^{2+}]_{cyt}$ responses to NaCl is markedly different from "drought" has been anticipated by Shi et al. (2002), who showed that root growth deprivation is different under NaCl and drought stress.

There are two differences between NaCl and "drought" $[Ca^{2+}]_{cyt}$ responses: First, the whole $[Ca^{2+}]_{cyt}$ is permanently shifted by NaCl toward a higher level (Figs. 8B and 9, black curves). Second, the short-term response to NaCl is prolonged when compared with the $[Ca^{2+}]_{cyt}$ kinetic under "drought" treatment (Fig. 8, C and D). In particular, these short-term differences are well in line with Knight et al. (1997b).

Prolonged $[Ca^{2+}]_{cyt}$ elevations have been seen with other studies where the cytoplasm has been challenged with an excess of other monovalent ions, namely H^+ (Plieth et al., 1997, 1999b). The main conclusion drawn in these studies is that calcium acts as protecting and ameliorative agent. Our assumption is that this can hold under Na^+ stress, too. The ameliorative action of calcium may result from various cellular interactions.

First, an increase of $[Na^+]$ also does affect cellular calcium buffer systems like H^+ ions do: Monovalent ions are able to displace stabilizing Ca^{2+} ions from their binding sites (i.e. phospho- and carboxy head groups in membranes, Dawson and Hauser, 1970; Hanson, 1984; and uronic groups in the cell wall, DeMarty et al., 1984). Thus, it makes sense for a plant cell to respond to a strong and dangerous increase of toxic monovalent ions with an active release of Ca^{2+} ions into both the cytoplasm (Figs. 8B and 9, black curves) and the apoplast (gray curves) to counteract loss of membrane and cell wall integrity even if enzyme regulation, signal transduction, and, thus, cellular activity are thereby negatively affected. This somewhat unspecific role could be designated as an "emergency brake function" of calcium. Because there is evidence for a calcium buffer capacity in the millimolar range in the cytoplasm (Plieth et al., 1997; Plieth and Hansen, 1998) and the apoplast (DeMarty et al., 1984; Smart and Trewavas, 1984), the increases of free $[Ca^{2+}]$ reported here (Figs. 8 and 9) constitute only a tiny percentage of the total amount of activated Ca^{2+} ions. Hence, the quantity of calcium mobilized by NaCl seems rather massive.

Second, one more specific effect of Ca^{2+} in the apoplast is its action on voltage-independent ions channels (VICs). VICs are believed to be the major doorway for Na^+ into the cell (Amtmann and Sanders, 1999). Because Ca^{2+} inhibits VIC-mediated Na^+ currents (White and Lemtiri-Chlieh, 1995; Maathius and Amtmann, 1999; White and Davenport, 2002); thus, it helps to establish a favorable intracellular K^+ to Na^+ ratio. Hence, calcium is an "ion transport modulator." This specific modulator function is not a matter of Ca^{2+} to Na^+ ratio in the extracellular matrix but rather a question of a critical external Ca^{2+} concentration necessary to be maintained for effective Na^+ influx inhibition (Davenport et al., 1997; Tyerman et al., 1997; Murata et al., 1998; Kinraide, 1999).

Third, Ca^{2+} ions have signaling properties. A calcineurin-like protein SOS3 has been identified to play a significant role in NaCl stress response in Arabidopsis (Liu and Zhu, 1998). SOS3 defective mutants are NaCl hypersensitive. This phenotype is partly suppressed by increased levels of external Ca^{2+}. Additional support for this Ca^{2+} calcineurin signaling pathway has been given by Pardo et al. (1998). Hence, calcium has a "signaling function." The current proposed regulatory pathway for intracellular Na^+ and K^+ homeostasis and Na^+ tolerance in plants assumes an increase in $[Ca^{2+}]_{cyt}$ under Na^+ stress (Halfter et al., 2000; Zhu, 2001) that is needed to switch kinases and phosphatases related to NaCl tolerance (Liu and Zhu, 1998; Pardo et al., 1998; Xiong and Zhu, 2002) and phosphoinositol turnover (Hirayama et al., 1995), which in turn is related to $[Ca^{2+}]$ signaling.

It has been shown that calcium activates cell wall phosphatases (DeMarty et al., 1984). Moreover, it has been reported several times that apoplastic calmodulin plays an important role in signal transduction (Sun et al., 1994, 1995; Tang et al., 1996; Ma and Sun, 1997; Ma et al., 1999). Thus, calcium seems to have a signaling function in the apoplast as well. However, despite all findings dedicating $[Ca^{2+}]_{apo}$ an important role in toxicity alleviation and NaCl stress response, no effect of external Ca^{2+} on Na^+ toxicity was found recently in Arabidopsis (Essah, 2000). Hence, no conclusive picture can be drawn yet of how Ca^{2+} is actually working in the apoplast in Arabidopsis.

Taken together, extracellular and cellular calcium perform different tasks. The experiments shown here provide the first information, to our knowledge, of how calcium behaves extracellularly and intracellularly in Arabidopsis under NaCl stress. Massive permanent Ca^{2+} shifts are broad-spectrum responses that may address unspecific protection mechanisms, ion transport, and specific signaling under NaCl stress circumstances.

MATERIALS AND METHODS

Plasmid Constructs

Standard PCR and cloning techniques (Sambrook et al., 1989; Ausubel et al., 1999) were employed to engineer the plasmid constructs described

below. For cytoplasmic expression, we started from a plasmid pCM2 (Moore, 2000), which is a pUC18 derivative equipped with a plant expression unit consisting of a short CaMV35S promoter and the corresponding terminator (528 and 193 bp, respectively; Pietrzak et al., 1986). pCM2 contains an in-frame fusion of the GFP gene (GFP5; Haseloff et al., 1997) downstream linked with AQ cDNA (Fig. 1A).

Because there is a *ClaI* site in GFP5 383 bp downstream of ATG, and all relevant mutations that render GFP pH sensitive are downstream of *ClaI*, we decided to exchange the second part of GFP5 between *ClaI* and *XhoI* with corresponding fragments produced by PCR amplification from ratiometric and ecliptic pHluorins (Miesenböck et al., 1998). In this way, pratioGFP:AQ and peclipGFP:AQ were constructed.

For cytoplasmic expression and better cellular distribution, we introduced the P99S mutation of *sm*GFP (soluble modified GFP) into the gene constructs by substituting the parts between *XbaI* and *ClaI* sites with the corresponding portion of *sm*GFP (CD3–026; Davis and Vierstra, 1998) obtained by PCR. Resulting plasmids were named p*sm*ratioGFP:AQ and p*sm*eclipGFP:AQ (Fig. 1, E and F).

For apoplastic targeting, we used the Arabidopsis chitinase signal sequence (22 amino acids) from plasmid p*BIN35S-mgfp5-ER*, which is known to target to the ER (Haseloff et al., 1997). Omitting the sequence coding for HDEL at the C-terminal end of the protein interrupts the continuous retrieval of the protein from the cis-Golgi to the ER lumen, causing secretion of the gene product and, thus, accumulation of the protein in the apoplast. The first part of the gene cassette in p*BIN35S-mgfp5-ER*, which contains a 66-bp sequence coding for a chitinase signal propeptide (22 amino acids) together with the first 383 bp of GFP sequence flanked by *XbaI* and a *ClaI* recognition sites, were generated by PCR. The PCR product was used to replace the corresponding *XbaI-ClaI* fragment in pCM2, and pH-sensitive mutations of ratiometric and ecliptic pHluorins were introduced as has been done with the cytoplasmic expression constructs, resulting in two plasmids: p*chi*ratioGFP:AQ and p*chi*eclipGFP:AQ. Finally, we replaced the AQ gene in these constructs with an engineered AQ with reduced Ca^{2+} affinity (LAAQ; Kendall et al., 1992) using *XhoI* and *PstI* restriction sites. Thus, two more gene constructs with a 66-bp apoplast signal sequence fused to pH and $[Ca^{2+}]$ indicator genes, namely p*chi*ratioGFP:LAAQ and p*chi*eclipGFP:LAAQ (Fig. 1, C and D), were produced. All gene constructs were confirmed by sequencing.

All gene cassettes (Fig. 1) were finally transferred to the binary vector pBI121 (Jefferson et al., 1987) using *XbaI* and *SacI*. This vector confers kanamycin resistance in plants and enables stable insertion of the genes into the Arabidopsis genome via *Agrobacterium tumefaciens*-mediated transformation (described below).

Bacterial Expression of Indicator Protein

To verify that modifications did not alter indicator properties, all indicator and fusion cDNAs were also cloned into bacterial expression vectors (pRSET; Invitrogen GmbH, Karlsruhe, Germany). Indicator proteins were expressed in bacteria and assessed fluorometrically (Figs. 2 and 3) and luminometrically. Because bacterial expression of GFPs is sometimes problematic (Gonzalez and Ward, 2000), and pRSET is leaky expressing when not induced, we grew bacteria under strong selection (70 $\mu g\ mL^{-1}$ chloramphenicol and 200 $\mu g\ mL^{-1}$ ampicillin) on "Terrific Broth" medium (no. 243820, DIFCO Laboratories, Detroit) for 8 h at 37°C/150 rpm. Protein production was induced by 1 mM isopropylthio-β-galactoside at 20°C/300 rpm overnight (i.e. 15 h). For protein isolation, bacteria were cracked in 200 mM phosphate buffer (pH 7.5) using either a French press or several freeze-thaw cycles. Debris was removed by centrifugation and filtering the supernatant through a 0.45-μm nylon filter. Cleared bulk protein extract was used for in vitro fluorescence assays at room temperature (Figs. 2 and 3) and appropriately buffered pH.

Plant Transformation

The floral dip method for *A. tumefaciens*-mediated transformation was used (Clough and Bent 1998; http://plantpath.wisc.edu/~afb/protocol.htm)to transform Arabidopsis of ecotype Columbia (Col0), Nottingham Arabidopsis Stock Centre no. N1092). Seeds from transformed plants were vapor phase sterilized in accordance with Clough and Bent (http://plantpath.wisc.edu/~afb/vapster.html) and sown on sterile solid medium (1× Murashige and Skoog, no. M0222, Duchefa, Haarlem, The Netherlands; and 0.75% [w/v] agar, no. A1296, Sigma, St. Louis) containing 50 $\mu g\ mL^{-1}$ kanamycin and supplemented with 1% (w/v) Suc. After 3 d at 4°C vernalization in the dark, plates were transferred to a growth chamber and cultivated at 70% relative humidity, 21°C, and 16-h photoperiod. Kanamycin-resistant F_1 transformants were transferred to soil in small pots, covered with ARACONs (BetaTech, Gent, Belgium), and further cultivated under same conditions. After selfing, plants of the F_2 generation were screened for fluorescence and luminescence. Lines with highest expression of the indicator fusion protein were selected, and individual F_2 plants were used for further proliferation into F_3 to find homozygous lines.

Plant Material

According to Figure 1, B to E, we obtained five transgenic lines expressing different indicator proteins: GFP5 and AQ in the apoplast (Fig. 1B), ratiometric pHluorin and LAAQ in the apoplast (Fig. 1C), ecliptic pHluorin and LAAQ in the apoplast (Fig. 1D), soluble modified ratiometric pHluorin and AQ in the cytoplasm (Fig. 1E), and soluble modified ecliptic pHluorin and AQ in the cytoplasm (Fig. 1F).

Plants were used for experiments when grown for 1 to 4 weeks on vertical 1.2% (w/v) plant agar (no. A1296, Sigma) supplemented with 0.5× Murashige and Skoog medium (no. M0222, Duchefa) and no sugar.

In Vivo Measurements of pH and $[Ca^{2+}]$

Because we used a double excitation fluorescence setup (described below), only Arabidopsis lines expressing ratiometric pHluorin were used in this study for pH measurements. For pH experiments, whole plants were placed in a plastic petri dish with a thin (0.13-mm) glass bottom. Only fully developed lateral branch roots were chosen and mechanically fixed to the glass bottom.

Solutions and Perifusion

NaCl stress was applied by 100 mM NaCl (Merck, Darmstadt, Germany). Drought stress was mimicked by application of isosmotic mannitol (Merck) solution (i.e. 200 mM). Osmolarities of both NaCl and mannitol solutions were checked by osmometry (CSMOMAT 030 Genotec GmbH, Berlin). All solutions were unbuffered, if not stated otherwise. During all experiments, plant material was rinsed with freshly prepared and aerated solutions. For pH measurements, the petri dish was perifused with a flow rate of 5 mL min^{-1}. For $[Ca^{2+}]$ measurements, the perfusion rate was 3 mL min^{-1} in the luminometer cuvette. Perifusion solutions of different composition were automatically switched by computer-controlled magnetic pinch solenoid valves (Sirai, Steinhöring, Germany).

pH Measurements

The dish with fixed plant material was mounted on an inverse microscope (Diaphot, Nikon, Düsseldorf, Germany) and perifused at all times with freshly made and aerated medium. The microscope was equipped with a 20× objective (Fluor PhSDL, Nikon) and an excitation ratio imaging device (TILL Photonics, Gräfelfing, Germany). GFP fluorescence was excited with light of 395, 428, and 475 nm from a monochromator (TILL), and emission was measured at 535 nm (emission filter, HQ535/50; dichroic mirror, 500dcxr; AHF, Tübingen, Germany). Fluorescent images were taken every 12 s. The F_{395} to F_{475} ratio was used as a measure for pH (calibration; Fig. 2B), and the F_{428} signal (i.e. isoexcitation point of ratio pHluorin; Fig. 2A) was taken as control to detect possible artifacts such as movement of the specimen or pH-sensitive autofluorescence. Macro programming of experiments, image acquisition, data collection, and evaluation were carried out using the software TillVision version 3.31 (TILL) extended with custom-tailored software for control of magnetic valves.

Reconstitution of AQ and Ca^{2+} Measurements

Reconstitution of AQ with CTZ was performed in planta essentially as described previously (Knight et al., 1997a). Synthetic native CTZ was from

Prolume Ltd. (Pittsburgh), and cp-CTZ was from Molecular Probes Europe (Leiden, The Netherlands). In brief, CTZ was dissolved first in methanol to have a 1 mM stock solution and then added to pure water to produce a 10 μM solution for reconstitution. Roots were dissected from plants grown on vertical agar and incubated in reconstitution medium in the dark. Roots with apoplastic expression lines were reconstituted for 3 to 4 h. Roots with cytoplasmic expression were reconstituted longer (i.e. overnight; 8–15 h) if not stated otherwise.

Reconstituted roots from about five plants were placed in a standard 4.5-mL acryl cuvette (no. 67.755; Sarstedt, Nümbrecht, Germany) equipped with inlet and outlet for perfusion. The cuvette was fixed in a purpose-built, light-tight sample housing in front of a chemiluminometer (PMT 9829A, Electron Tubes Ltd., Ruislip, UK). Luminescence of the specimen was integrated every 12 s.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes.

ACKNOWLEDGMENTS

We thank Gero Miesenböck and James Rothman (Memorial Sloan-Kettering Cancer Centre, New York) for the generous gift of pHluorin cDNA, Cathy Moore (Oxford University) for the generous gift of plasmid pCM2, Jim Haselhoff (Cambridge University, UK) for the binary vector pBINm-*gfp5-ER*, and Markus Böttcher (Kiel University, Germany) for technical assistance with CLSM.

Received August 28, 2003; returned for revision October 2, 2003; accepted November 25, 2003.

LITERATURE CITED

Amtmann A, Sanders D (1999) Mechanisms of Na^+ uptake by plant cells. Adv Bot Res **29:** 76–112

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1999) Short Protocols in Molecular Biology. Wiley, New York

Blatt MR, Grabov A (1997) Signal redundancy gates and integration in the control of ion channels for stomatal movement. J Exp Bot **48:** 529–537

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J **16:** 735–743

Cramer GR, Jones RL (1996) Osmotic stress and abscisic acid reduce cytosolic calcium activities in roots of *Arabidopsis thalisna*. Plant Cell Environ **19:** 1291–1298

Dannel F, Pfeffer H, Marschner H (1995) Isolation of apoplasmic fluid from sunflower leaves and its use for studies on influence of nitrogen supply on apoplasmic pH. J Plant Physiol **146:** 273–278

Davenport RJ, Reid RJ, Smith FA (1997) Sodium-calcium interactions in two wheat species differing in salinity tolerance. Physiol Plant **99:** 323–327

Davis SJ, Vierstra RD (1998) Soluble, high fluorescent variants of green fluorescent protein (GFP) for use in higher plants. Plant Mol Biol **36:** 521–528

Dawson RMC, Hauser H (1970) Binding of calcium to phospholipids. *In* AW Cuthbert, ed, Calcium and Cellular Function. MacMillan and Co. Ltd., London, 17–43

DeMarty M, Morvan C, Thellier M (1984) Calcium and the cell wall. Plant Cell Environ **7:** 441–448

Di Sansebastiano GP, Paris N, Marc-Martin S, Neuhaus J-M (2001) Regeneration of a lytic central vacuole and of neutral peripheral vacuoles can be visualized by green fluorescent protein targeted to either type of vacuoles. Plant Physiol **126:** 78–86

Essah PA (2000) Sodium transport in *Arabidopsis thaliana*. PhD thesis. University of Cambridge, UK

Felle HH (1987) Proton transport and pH control in *Sinapis alba* root hairs: a study carried out with double barreled pH microelectrodes. J Exp Bot **340:** 354–363

Felle HH (1998) The apoplast pH of the *Zea mays* root cortex as measured with pH-sensitive microelectrodes: aspects of regulation. J Exp Bot **49:** 987–995

Felle HH (2001) pH: signal and messenger in plant cells. Plant Biol **3:** 577–591

Frigerio L, Ombretta F, Felipe DH, Neuhaus J-M, Vitale A (2001) the C-terminal tetrapeptide of phaseolin is sufficient to target green fluorescent protein to the vacuole. J Plant Physiol **158:** 400–503

Gilroy S, Trewavas A (1994) A decade of plant signals. BioEssays **16:** 677–682

Gonzalez DG, Ward WW (2000) Large scale purification of recombinant green fluorescent protein from *Escherichia coli*. Methods Enzymol **305:** 212–223

Halfter U, Ishitani M, Zhu J-K (2000) The *Arabidopsis* SOS2 protein kinase physically interacts with and is activated by the calcium-binding protein SOS3. Proc Natl Acad Sci USA **97:** 3735–3740

Hanson JB (1984) The function of calcium in plant nutrition. *In* PB Tinker, A Läuchli, ed, Advances in Plant Nutrition. Praeger Special Studies, Praeger Scientific, New York pp 149–208

Haseloff J, Siemering KR, Prasher DC, Hodge S (1997) Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. Proc Natl Acad Sci USA **94:** 2122–2127

Hawes C, Saint-Jore C, Martin B, Zheng H-Q (2001) ER confirmed as the location of mystery organelles in *Arabidopsis* plants expressing GFP. Trends Plant Sci **6:** 245–246

Hirayama T, Ohto C, Mizoguchi T, Shinozaki K (1995) A gene encoding a phosphatidylinositol-specific phospholipase C is induced by dehydration and salt stress in *Arabidopsis thaliana*. Proc Natl Acad Sci USA **92:** 3903–3907

Hoffmann B, Kosegarten H (1995) FITC-dextran for measuring apoplast pH and apoplast pH gradients between various cell types in sunflower leaves. Physiol Plant **95:** 327–335

Hoson T (1998) Apoplast as site of response to environmental signals. J Plant Res **111:** 167–177

Jefferson RA, Kavanaugh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J **6:** 3901–3907

Jones HE, Holland IB, Campbell AK (2002) Direct measurement of free Ca^{2+} shows different regulation of Ca^{2+} between the periplasm and the cytosol of *Escherichia coli*. Cell Calcium **32:** 183–192

Kendall JM, Sala-Neby G, Ghalaut V, Dormer RL, Campbell AK (1992) Engineering the Ca^{2+}-activated photoprotein aequorin with reduced affinity for calcium. Biochem Biophys Res Commun **187:** 1091–1097

Kiegle E, Moore CA, Haseloff J, Tester J, Knight MR (2000) Cell-type-specific calcium responses to drought salt and cold in *Arabidopsis* roots. Plant J **23:** 267–278

Kinzaide TB (1999) Interactions among Ca^{2+}, Na^+, and K^+ in salinity toxicity: quantitative resolution of multiple toxic and ameliorative effects. J Exp Bot **50:** 1495–1505

Knight H (2000) Calcium signaling during abiotic stress in plants. Int Rev Cytol **195:** 269–324

Knight H, Trewavas AJ, Knight MR (1997a) Recombinant aequorin methods for measurement of intracellular calcium in plants. Plant Mol Biol Man **C4:** 1–22

Knight H, Trewavas AJ, Knight MR (1997b) Calcium signaling in *Arabidopsis thaliana* responding to drought and salinity. Plant J **12:** 1067–1078

Knight MK, Campbell AK, Smith SM, Trewavas AJ (1991) Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. Nature **352:** 524–526

Kosegarten H, Englisch G (1994) Effect of various nitrogen forms on the pH of leaf apoplast and on iron chlorosis of *Glycine max* L. Z Pflanzener Bodenkd **157:** 401–405

Liu J, Zhu J-K (1998) A calcium sensor homolog required for plant salt tolerance. Science **280:** 1943–1945

Lohaus G, Pennewis K, Sattelmacher B, Hussmann M, Mühling KH (2001) Is the infiltration-centrifugation technique appropriate for the isolation of apoplastic fluid? A critical evaluation with different plant species. Physiol Plant **111:** 457–465

Lynch J, Polito VS, Läuchli A (1989) Salinity stress increases cytoplasmic Ca^{2+} activity in maize root protoplasts. Plant Physiol **90:** 1271–1274

Ma L, Sun D-Y (1997) The effects of extracellular calmodulin on initiation of *Hippeastrum rutilum* pollen germination and tube growth. Planta **202:** 336–340

Ma L, Xu X, Cui S, Sun D (1999) The presence of a heterotrimeric G protein and its role in signal transduction of extracellular calmodulin in pollen germination and tube growth. Plant Cell **11:** 1351–1363

Maathius FJM, Amtmann A (1999) K^+ nutrition and Na^+ toxicity: the basis of cellular K^+/Na^+ ratios. Ann Bot **84:** 123–133

Miesenböck G, Angelis DA de, Rothman JE (1998) Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent protein. Nature **394:** 192–195

Moore CA (2000) An investigation into cellular and molecular aspects of calcium-based specificity of signalling in *Arabidopsis thaliana*. PhD thesis. University of Oxford

Moseyke N, Feldman LJ (2001) Expression of pH-sensitive green fluorescent protein in *Arabidopsis thaliana*. Plant Cell Environ **24:** 557–563

Mühling K-H, Plieth C, Hansen U-P, Sattelmacher B (1995) Apoplastic pH of intact leaves of *Vicia faba* as influenced by light. J Exp Bot **46:** 377–382

Mühling KH, Sattelmacher B (1995) Apoplastic ion concentration of intact leaves of field bean (*Vicia faba*) as influenced by ammonium and nitrate nutrition. J Plant Physiol **147:** 81–86

Murata Y, Fujita M, Nakatani T, Obi I, Kakutani T (1998) Effect of Na^+ on Ca^{2+} binding on the plasma membrane of barley mesophyll cells: an electrophoretic study. Plant Cell Physiol **39:** 452–457

Neuhaus J-M (2000) GFP as a marker for vacuoles in plants. *In* DG Robinson, ed, Annual Plant Reviews: Vacuolar Compartments. Sheffield Academic Press, Sheffield, England, pp 254–269

Pardo JM, Reddy MP, Yang S, Maggio A, Huh G-H, Matsumoto T, Coca MA, Paino-D'Urzo M, Koiea H, Yun D-J et al. (1998) Stress signaling through Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin mediates salt adaptation in plants. Proc Natl Acad Sci USA **95:** 9681–9686

Pauly N, Knight MR, Thuleau P, Graziana A, Muto S, Ranjeva R, Mazars C (2001) The nucleus together with the cytosol generates patterns of specific cellular calcium signatures in tobacco suspension culture cells. Cell Calcium **30:** 413–421

Persson S, Love J, Tsou P-L, Robertson D, Thompson WF, Boss WF (2002) When a day makes a difference. Interpreting data from endoplasmic reticulum-targeted green fluorescent protein in cells grown in suspension culture. Plant Physiol **128:** 341–344

Pietrzak M, Shillito RD, Hohn T, Potrykus I (1986) Expression in plants of two bacterial antibiotic genes after protoplast transformation with a new plant expression vector. Nucleic Acids Res **14:** 5857–5868

Plieth C (2001) Plant calcium signaling and monitoring: pros and cons and recent experimental approaches. Protoplasma **218:** 1–23

Plieth C, Hansen U-P (1996) Methodological aspects of pressure loading of Fura-2 into *Characean* cells. J Exp Bot **47:** 1601–1612

Plieth C, Hansen U-P (1998) Cytoplasmic Ca^{2+} and H^+ buffers in green algae: a reply. Protoplasma **203:** 210–213

Plieth C, Hansen U-P, Knight H, Knight MR (1999a) Temperature sensing by plants: the primary mechanisms of signal perception and calcium response. Plant J **18:** 491–497

Plieth C, Sattelmacher B, Hansen U-P (1997) Cytoplasmic Ca^{2+}-H^+-exchange buffers in green algae. Protoplasma **198:** 107–124; erratum **Plieth C, Sattelmacher B, Hansen U-P** (1997) Protoplasma **199:** 223

Plieth C, Sattelmacher B, Hansen U-P, Knight MR (1999b) Low pH-mediated elevations in cytosolic calcium are inhibited by aluminium: a potential mechanism for aluminium toxicity. Plant J **18:** 643–650

Plieth C, Sattelmacher B, Trewavas AJ, Hansen UP, Knight MR (2001) Engineering plants expressing calcium and pH indicators in the cytoplasm and the apoplast. *In* WJ Horst, ed, Plant Nutrition: Food Security and Sustainability of Agro-Ecosystems through Basic and Applied Research, Proceedings of XIV International Plant Nutrition Colloquium, Hannover, Germany, 2001, Developments in Plant and Soil Sciences Series, Vol 92. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 252–253

Plieth C, Trewavas AJ (2002) Reorientation of seedlings in the earth's gravitational field induces cytosolic calcium transients. Plant Physiol **129:** 786–796

Roos W (2000) Ion mapping in plant cells: methods and applications in signal transduction research. Planta **210:** 347–370

Sakurai N (1998a) Dynamic function and regulation of apoplast in the plant body. J Plant Res **111:** 133–148

Sakurai N (1998b) Dynamism of apoplast. J Plant Res **111:** 131

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sanders D, Brownlee C, Harper JF (1999) Communicating with calcium. Plant Cell **11:** 691–706

Sanders D, Pelloux J, Brownlee C, Harper JF (2002) Calcium at the crossroads of signaling. Plant Cell **14:** S401–S417

Scrase-Field SAMG, Knight MR (2003) Calcium: just a chemical switch? Curr Opin Plant Biol **6:** 500–506

Shi H, Quintero FJ, Pardo JM, Zhu J-K (2002) The putative plasma membrane Na^+/H^+ antiporter SOS1 controls long distance Na^+ transport in plants. Plant Cell **14:** 465–477

Smart CC, Trewavas AJ (1984) Abscisic acid-induced turion formation in *Spirodela polyrrhiza*: IV. Comparative ion flux characteristics. Plant Cell Environ **7:** 521–531

Sun DY, Li HB, Cheng G (1994) Extracellular calmodulin accelerates the proliferation of suspension-cultured cells of *Angelica dahurica*. Plant Sci **99:** 1–8

Sun DY, Bian YQ, Zhao BH, Zhao LY, Yu XM, Shengjun D (1995) The effects of extracellular calmodulin on cell wall regeneration of protoplasts and cell division. Plant Cell Physiol **36:** 133–138

Takahashi K, Isobe M, Knight MR, Trewavas AJ, Muto S (1997) Hypoosmotic shock induces increase in cytosolic Ca^{2+} in tobacco suspension-culture cells. Plant Physiol **113:** 587–594

Tang J, Wu S, Bai J, Sun D-Y (1996) Extracellular calmodulin-binding proteins in plants: purification of a 21-kDa calmodulin-binding protein. Planta **198:** 510–516

Taylor DP, Slattery J, Leopold AC (1996) Apoplastic pH in corn root gravitropism: a laser scanning confocal microscopy measurement. Physiol Plant **97:** 35–38

Trewavas AJ, Malho R (1998) Ca^{2+} signalling in plant cells: the big network! Curr Opin Plant Biol **1:** 428–433

Tyerman SD, Skerrett M, Garrill A, Findlay GP, Leigh RA (1997) Pathway for the permeation of Na^+ and Cl^- into protoplasts derived from the cortex of wheat roots. J Exp Bot **48:** 459–480

Ward JM, Pei Z-M, Schroeder JI (1995) Roles of ion channels in initiation of signal transduction in higher plants. Plant Cell **7:** 833–844

White PJ, Davenport RJ (2002) The voltage-independent cation channel in the plasma membrane of wheat roots is permeable to divalent cations and may be involved in cytosolic Ca^{2+} homeostasis. Plant Physiol **130:** 1386–1395

White PJ, Lemtiri-Chlieh F (1995) Potassium currents across the plasma membrane of protoplasts derived from rye roots: a patch-clamp study. J Exp Bot **46:** 497–511

Xiong L, Zhu J-K (2002) Salt Tolerance. The Arabidopsis Book. American Society of Plant Biologists. www.aspb.org/publications/arabidopsis/index.cfm

Zhu J-K (2001) Cell signaling under salt, water and cold stresses. Curr Opin Plant Biol **4:** 401–406

Zieschang HE, Köhler K, Sievers A (1993) Changing proton concentrations at the surface of gravistimulated *Phleum* roots. Planta **190:** 546–554